Remarks

Rejection Under 35 U.S.C. § 103

Claims 1-26, 29 and 30 were rejected under 35 U.S.C. § 103(a) as being unpatentable over U.S. Patent No. 5,750,848 to Kruger, et al. ("Kruger"). Applicants respectfully traverse this rejection.

The Legal Standard

The U.S. Patent and Trademark Office has the burden under 35 U.S.C. § 103 to establish a *prima facie* case of obviousness. *In re Warner et al.*, 379 F.2d 1011, 154 U.S.P.Q. 173, 177 (C.C.P.A. 1967); *In re Fine,* 837 F.2d 1071, 1074, 5 U.S.P.Q.2d 1596, 1598-99 (Fed. Cir. 1988). **To establish a *prima facie* case of obviousness, three basic criteria must be met.** First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art and not based on applicant's disclosure. *In re Vaeck,* 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

Claims for an invention are not *prima facie* obvious if the primary references do not suggest all elements of the claimed invention and the prior art does not suggest the modifications that would bring the primary references into conformity with the application claims. *In re Fritch*, 23 U.S.P.Q.2d, 1780 (Fed. Cir. 1992); *In re Laskowski*, 871 F.2d 115 (Fed. Cir. 1989).

This is not possible when the claimed invention achieves more than what any or all of the prior art references allegedly suggest, expressly or by reasonable implication.

The Factual and Legal Analysis

1. The Examiner has failed to individually examine the claims.

The claims are drawn to:

A bacteria or plant that makes PHA, genetically engineered so that it can make the PHA through a fatty acid biosynthetic pathway (claims 1-12 and 29)

A method of engineering a bacteria or plant that makes PHA so that it can make the PHA through a fatty acid biosynthetic pathway (claims 13-19 and 29)

A method of making medium chain length PHA using the organism of claims 1-12 (claims 20-24

The invention is to start with an organism that makes PHA, either a bacteria or a plant, then engineer the organism so that it expresses:

(1) 3-hydroxyacyl-ACP thioesterase AND

(2) A PHA synthease that incorporates medium chain length hydroxy acids

OR medium chain length 3-hydroxy fatty acid acyl CoA synthetase,

So that the bacteria or plant accumulates ***medium chain PHA through the fatty acid biosynthesis pathway.***

The analysis for a composition claim is not the same as the method for making nor the method of using the composition. The examiner has failed to examine the claims noting these differences but has instead focused on a single common element, whether or not the organism (a bacteria, not a plant, as defined not only by the independent claims, but defined solely by claims 8, 9, 17, 18, and 25).

2. Inherency is only an issue of novelty; not the basis for an obviousness rejection.

It is well established that inherency is only relevant to novelty, not obviousness. One cannot find something obvious from an alleged inherent disclosure. The Federal Circuit has clearly set forth the standard for evaluating inherency in *In re Robertson*, 49 U.S.P.Q. 1949 (Fed. Cir. 1999). The court held that a claim element is not "inherent" in the disclosure of a prior art reference unless extrinsic evidence clearly shows that missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill in the art. "Inherency, however, may not be established by mere probabilities or possibilities" (49 U.S.P.Q. at 1950-51). The doctrine of inherency has undergone significant clarification in recent years. The courts have consistently held that proof of inherency is not met by a mere showing of a possibility or probability that the missing element or function is present. *Trintec Indus., Inc. v. Top-U.S.A. Corp.*, 295 F.3d 1292, 1295 (Fed.Cir.2002); *Continental Can Co. v. Monsanto Co.*, 948 F.2d 1264, 1269 (Fed.Cir.1991).

In the present case, the examiner has acknowledged that Kruger does not disclose each of the claimed elements since the rejection is made under 35 U.S.C. 103, not 102. Therefore inherency is not an issue. It is clear that to establish a rejection under 35 U.S.C. § 103 the cited references must (1) recite each element of the claims, (2) provide one of skill in the art with the motivation to modify the cited reference as applicants have done and (3) provide one of ordinary skill in the art with a reasonable expectation of success.

3. Kruger does not disclose each claimed element, nor provide the motivation for one skilled in the art to combine and use as claimed.

Kruger describes a single isolated gene, phaG from *Pseudomonas putida*, which is alleged to encode a 3-hydroxyacyl-ACP-CoA transferase activity useful for producing (D)-3-hydroxyacyl-CoA precursors for the biosynthesis of polyhydroxyalkanoate (PHA) when grown on simple carbon sources (see col. 3, lines 1-15).

As noted above, the claims require the bacteria or plant express the following transgenes:

(1) 3-hydroxyacyl-ACP thioesterase AND

(2) A PHA synthease that incorporates medium chain length hydroxy acids OR medium chain length 3-hydroxy fatty acid acyl CoA synthetase,

(1) Recite Each Element Of The Claims

Kruger does not disclose or suggest transgenic organisms that express (1) a transgene encoding an enzyme having the catalytic activity of 3-hydroxyacyl-ACP thioesterase **and** (2) one or more enzymes having the catalytic activity of acyl-CoA synthetase or acyl CoA transferase,

So that the organisms accumulate medium chain length polyhydroxyalkanoates through the fatty acid biosynthesis pathway.

Kruger may disclose engineering an organism to express an acyl CoA transferase. See col. 3, lines 20-24. However, Kruger does not disclose, nor suggest, ALSO engineering an organism with either an enzyme having the catalytic activity of acyl-CoA synthetase or acyl CoA transferase. It is not enough that a gene may have been isolated which, armed with applicants' disclosure, one could deduce encoded such an enzyme and that, again in hindsight, combine with the gene encoding the acyl CoA transferase, and then again using hindsight, not the teaching of Kruger, provide appropriate short chain substrate for utilization in the fatty acid pathway.

The examiner has decided, with no support in Kruger, that because the term "phaG" is used, it must encompass the disclosure of the two claimed transgenes. However, there is no support for this conclusion.

The Examiner's attention is drawn to the discussion on page 7 of the application. This review of the various enzyme activities, and the examples, demonstrates that "unexpectedly", PhaG encodes only 3-hydroxy acyl ACP thioesterase (page 7, lines 8-12). The examples support the discussion that one *also needs* acyl CoA synthetase activity separate from this PhaG activity, in order to utilize the fatty acid biosynthetic pathway.

(2) Provide One of Skill in the Art with the Motivation to Modify the Reference and Provide One of Skill in the Art with a Reasonable Expectation of Success

Kruger states in column 3, lines 20-23, that methods are described for identifying genes that encode CoA-ACP acyltransferase (*phaG*) that would be useful in the **direct** conversion of acyl-ACP to acyl-CoA for PHA biosynthesis. According to Figure 1 in Kruger, there would be no need for a skilled artisan to combine *phaG* with any enzyme other than the enzymes involved in PHA biosynthesis, in any organism. Kruger does not recognize the need for an acyl synthetase or acyl transferase to supplement *phaG* in the production of medium chain PHA in E. coli. A skilled artisan reading the disclosure in Kruger, together with the disclosure that E3 (*phaG*) enabled P. *pupita* Kt2440 $PHAG_N$ 195 mutants (which are defective exclusively in the branch of PHA biosynthesis occurring via *de novo* fatty acid biosynthesis) to accumulate PHA (as evidenced by the opacity of the transductant colonies) would have no motivation to combine *phaG* with any other enzyme, other than an enzyme in the PHA biosynthetic pathway. Nowhere in Kruger is it taught that *phaG* transformed into *E. coli* as fragment E3 enabled the cells to accumulate PHA.

The composition of PHA depends on the PHA synthase, the carbon source, and the metabolic routes used. PHA's can be divided into two groups according to the length of their side chains- those with short side chains such as polyhydroxybutyric acid and those with **medium** length side chains. In bacteria, each PHA group is produced by a specific pathway. The medium chain length pendant groups PHAs are produced by PHA synthases which have substrate specificity favoring the larger C6-C14 monomeric units (please see the specification at page 2, lines 5-9). Kruger does not enable one of ordinary skill in the art to produce **medium** chain length PHAs in bacteria or plants. Kruger discloses that *phaG* can be used in conjunction with PHA synthase to produce PHA. However, Applicants have demonstrated at least at Example 2, that expression of *phaG* and a PHA synthase in bacteria did **not** result in PHA production. Similarly, Rhem, et al., *J. Biol. Chem.*, 273(37):24044-51 (1998) (attached, please see page 24048) investigated the functional expression of *phaG* in E. coli expressing phaC1, and observed no PHA accumulation in cells grown on glucose.

As defined by the claims, and as demonstrated by Applicants at least in Example 4, there is a requirement for supplemental acyl CoA synthetase or acyl CoA transferase activity for polymer production. Example 4 demonstrates that *phaG* acts ***in vivo*** as a 3-hydroxyacyl ACP thioesterase and not as a 3-hydroxyacyl-ACP-CoA transferase as suggested by Kruger.

Although Kruger discloses in column 14, lines 2-14 that an enzyme or a combination of enzymes that converts β-hydroxy-ACP to B-hydoxyacyl-CoA could potentially be cloned by transforming a PHA-negative bacterium harboring only PHA synthase with a genomic library constructed from an organism suspected to have the desired activity, and then using the β-hydroxyl-CoA directly as a substrate for PHA synthesis by the PHA polymerase from a single carbon source, this is **not** recognition that one would need to have both *phaG* **and** a CoA-acyl

transferase or CoA-acyl synthetase in *E. coli* or plants. This is evidenced by the fact that Kruger discloses in the same paragraph that any strain making PHA would then be checked to determine if PHA synthesis is due to cloning of an acyltransferase (this is referring to CoA-ACP acyltransferase; please see discussion in column 12), a thioesterase plus ligase, or β-ketothiolase plus acyl-CoA reductase. It is clear that Kruger does not refer to a scenario wherein a CoA-ACP acyltransferase **plus** a CoA-transferase or synthetase for example are required together, because Kruger does not recognize that CoA-ACP acyltransferase can function solely as a thioesterase. This is further supported by the disclosure in column 2, lines 32-36, that the hydroxyl residues of the acyl-carrier proteins are converted to the corresponding CoA-derivatives, and this can be mediated in a one-step reaction by an (R)-3-hydroxyacyl (ACP to CoA) transferase (*phaG*).

The Examiner's assertion that the CoA-ACP acyltransferase disclosed in Kruger would inherently have thioesterase activity is without support. "In relying upon the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art." *Ex parte Levy*, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990). The Examiner has provided no facts or technical reasoning to support her determination that the thioesterase activity of CoA-ACP acylransferase *necessarily flows* from the teachings in Kruger. The Examiner also asserted that Kruger teaches that *phaG* can be used in conjunction with other PHA biosynthetic enzymes. However, from this teaching, one of ordinary skill in the art would expect that *phaG* can be used in conjunction with β-ketothiolase, 3-ketoacyl-CoA reductase and PHA synthase, the PHA biosynthetic enzymes known in the art, and also depicted in Figure 1 in Kruger.

In contrast, Applicants use *phaG* in combination with acyl-CoA synthetase or transferase, which is **not** a PHA biosynthetic enzyme. This is due to the discovery by Applicants that *phaG* in conjunction with other PHA biosynthetic enzymes would not result in the accumulation of PHA's in *E. coli*, since *phaG* alone was unable to provide the substrates for β-ketothiolase.

Since Kruger does not recognize the need for an acyl-transferase or synthetase in conjunction with *phaG*, Kruger cannot make obvious the claims.

Allowance of claims 1, 3-13, 15-20, 22-26, 29, and 30 is respectfully solicited.

Respectfully submitted,

Patrea L. Pabst
Reg. No. 31,284

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PABST PATENT GROUP LLP
400 Colony Square, Suite 1200
1201 Peachtree Street
Atlanta, Georgia 30361
(404) 879-2151
(404) 879-2160 (Facsimile)

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A New Metabolic Link between Fatty Acid *de Novo* Synthesis and Polyhydroxyalkanoic Acid Synthesis

THE *PHAG* GENE FROM *PSEUDOMONAS PUTIDA* KT2440 ENCODES A 3-HYDROXYACYL-ACYL CARRIER PROTEIN-COENZYME A TRANSFERASE*

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Bernd H. A. Rehm, Niels Krüger, and Alexander Steinbüchel‡

From the Institut für Mikrobiologie, Westfälische Wilhelms-Universität Münster, Corrensstraße 3, D-48149, Münster, Germany

To investigate the metabolic link between fatty acid *de novo* synthesis and polyhydroxyalkanoic acid (PHA) synthesis, we isolated mutants of *Pseudomonas putida* KT2440 deficient in this metabolic route. The gene *phaG* was cloned by phenotypic complementation of these mutants; it encoded a protein of 295 amino acids with a molecular mass of 33,876 Da, and the amino acid sequence exhibited 44% amino acid identity to the primary structure of the *rhlA* gene product, which is involved in the rhamnolipid biosynthesis in *Pseudomonas aeruginosa* PG201. S_1 nuclease protection assay identified the transcriptional start site 239 base pairs upstream of the putative translational start codon. Transcriptional induction of *phaG* was observed when gluconate was provided, and PHA synthesis occurred from this carbon source. No complementation of the *rhlA* mutant *P. aeruginosa* UO299-harboring plasmid pBHR81, expressing *phaG* gene under *lac* promoter control, was obtained. Heterologous expression of *phaG* in *Pseudomonas oleovorans*, which is not capable of PHA synthesis from gluconate, enabled PHA synthesis on gluconate as the carbon source. Native recombinant PhaG was purified by native polyacrylamide gel electrophoresis from *P. oleovorans*-harboring plasmid pBHR81. It catalyzes the transfer of the acyl moiety from *in vitro* synthesized 3-hydroxydecanoyl-CoA to acyl carrier protein, indicating that PhaG exhibits a 3-hydroxyacyl-CoA-acyl carrier protein transferase activity.

Fluorescent pseudomonads belonging to the rRNA homology group I are able to synthesize and accumulate large amounts of polyhydroxyalkanoic acids (PHA)[1] consisting of various saturated 3-hydroxy fatty acids with carbon chain length ranging from 6 to 14 carbon atoms as carbon and energy storage compound (1). PHA isolated from these bacteria contained also constituents with double bonds or with functional groups such as branched, halogenated, aromatic, or nitrile side chains (2). The composition of PHA depends on the PHA synthases, the carbon source, and the involved metabolic routes (2–6). In *Pseudomonas putida* at least three different metabolic routes occur for the synthesis of 3-hydroxyacyl coenzyme A thioesters, which are the substrates of the PHA synthase (7). (i) β-Oxidation is the main pathway when fatty acids are used as carbon source. (ii) Fatty acid *de novo* biosynthesis is the main route during growth on carbon sources that are metabolized to acetyl-CoA, like gluconate, acetate, or ethanol. (iii) Chain elongation reactions in which acetyl-CoA moieties are condensed to 3-hydroxyacyl-CoA is involved in the PHA synthesis during growth on hexanoate. Recently, recombinant PHA_{MCL} (MCL = medium chain length) synthesis was also obtained in a β-oxidation mutant of *Escherichia coli* LS1298 (*fadB*) expressing PHA synthase genes from *Pseudomonas aeruginosa* (8, 9), indicating that the β-oxidation pathway in *E. coli* provides precursors for PHA synthesis (8). From extended homologies of the primary structures of PHA_{MCL} synthases to PHA_{SCL} (SCL = short chain length) synthases (1), which occur in bacteria accumulating poly(3-hydroxybutyric acid) such as *e.g. Alcaligenes eutrophus*, it seems also likely that the substrate of PHA_{MCL} synthases is (*R*)-3-hydroxyacyl-CoA in pseudomonads. The main constituent of PHA of *P. putida* KT2442 from unrelated substrates such as gluconate is (*R*)-3-hydroxydecanoate (7, 10, 11). Thus, to serve as substrate for the PHA synthase, (*R*)-3-hydroxyacyl-ACP must be converted to the corresponding CoA derivative. This can be mediated in a one step reaction by an (*R*)-3-hydroxyacyl (ACP to CoA) transferase. Another possibility is the release of (*R*)-3-hydroxydecanoic acid by a thioesterase, and subsequent activation to the CoA derivative. Only few enzymes have been described catalyzing a similar reaction. Examples are the malonyl-CoA-ACP transferase, which catalyzes the transfer of the malonyl moeity from CoA to ACP (12), and (*R*)-3-hydroxydecanoyl-ACP-dependent UDP-GlcNAc acyltransferase, which catalyzes the transfer of hydroxydecanoyl moeity from ACP to UDP-GlcNAc (13, 14). In this study, we describe the isolation and characterization of *P. putida* KT2440 mutants, which are defective in the PHA synthesis via fatty acid *de novo* biosynthesis, and we identified and characterized the gene locus, which phenotypically complements these mutants. The gene product of *phaG* was purified, and the catalyzed reaction was identified.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth of Bacteria—Pseudomonads and *Escherichia coli* strains as well as the plasmids used in this study are listed in Table I. *E. coli* was grown at 37 °C in Luria-Bertani (LB) medium. Pseudomonads were grown at 30 °C either in nutrient broth complex medium (0.8%, w/v) or in a mineral salts medium with 0.05% (w/v) ammonia (15).

Nitrosoguanidine Mutagenesis—Mutagenesis was performed according to Miller (16). Cells were incubated for 15 min in the presence of 200 μg of *N*-methyl-*N'*-nitro-*N*- nitrosoguanidine/ml.

Polyester Analysis—3–5 mg of lyophilized cell material was subjected

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF053507.

‡ To whom correspondence should be addressed. Tel.: 49 251 833 9821; Fax: 49 251 833 8388.

[1] The abbreviations used are: PHA, polyhydroxyalkanoic acid; ACP, acyl carrier protein; PAGE, polyacrylamide gel electrophoresis; CDW, cellular dry weight; kbp, kilobase pair(s); ORF, open reading frame; HPLC, high performance liquid chromatography.

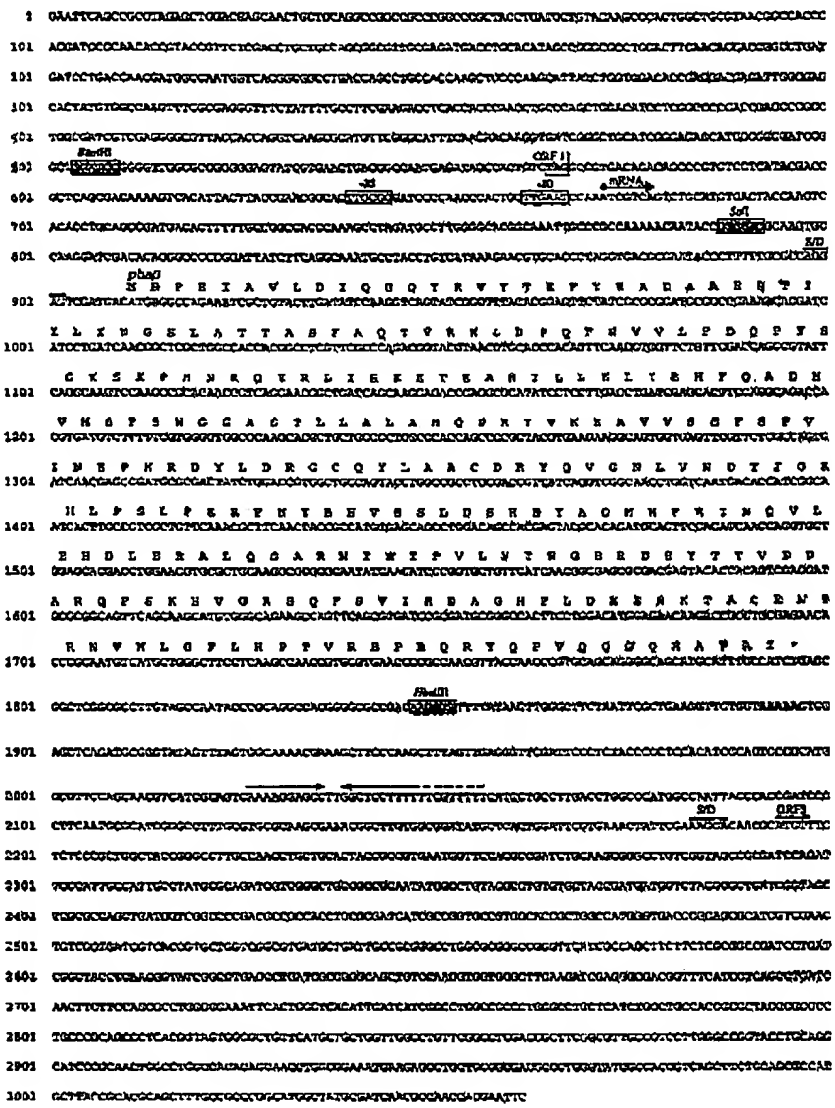

FIG. 1. **Nucleotide sequence of fragment E8.** Amino acids deduced from the nucleotide sequence are specified by standard one-letter abbreviations. The promoter sequence ("−10" and "−35") is *boxed*. Putative ribosome binding sites are indicated by *black bars* and the letters *SD*. The position of a tentative factor-independent transcriptional terminator downstream of *phaG* is indicated by *arrows*. An *arrow* starting with a *dot* indicates the transcription start site and direction of transcription.

to methanolysis in the presence of 15% (v/v) sulfuric acid. The resulting methyl esters of the constituent 3-hydroxyalkanoic acids were assayed by gas chromatography according to Brandl *et al.* (17) and as described in detail recently (10).

Isolation, Analysis, and Manipulation of DNA—Plasmid DNA was prepared from crude lysates by the alkaline extraction procedure (18). Total genomic DNA was isolated according to Ausubel *et al.* (19). All genetic procedures and manipulations of DNA were conducted as described by Sambrook *et al.* (20). DNA sequencing was carried out by the dideoxy chain termination method (21) with single-stranded or with double-stranded alkali-denatured plasmid DNA but with 7-deaza-guanosine 5′-triphosphate instead of dGTP (22) and with α-^{35}S-dATP using a T7 polymerase sequencing kit according to the manufacturer's protocol (Amersham Pharmacia Biotech). Synthetic oligonucleotides were used as primers, and the "primer-hopping strategy" (23) was employed. Analysis was done in 8% (w/v) acrylamide gels in buffer, pH 8.3, containing 100 mM hydrochloride, 83 mM boric acid, 1 mM EDTA, and 42% (w/v) urea in a S2-sequencing apparatus (Life Technologies, Inc.). Nucleic acid sequence data and deduced amino acid sequences were analyzed with the sequence analysis software package (version 6.2, June 1990) according to Devereux *et al.* (24). The nucleotide and amino acid sequence data reported here have been submitted to GenBank™ under accession number AF052507.

Determination of the Transcriptional Start Site—Total RNA was isolated as described by Oelmüller *et al.* (25). The determination of the transcriptional start site was done by a S_1 nuclease protection assay. The hybridization conditions for the S_1 nuclease protection assays were done as described by Berk and Sharp (26) and Sambrook *et al.* (20), and the S_1 nuclease reactions were conducted as described by Aldea *et al.* (27). DNA probes and dideoxynucleotide sequencing reactions for sizing the signals were performed with pBluescript SK⁻BH18 DNA as a template. In the annealing reaction, the oligonucleotide (5′-GGGTAT-TCGCGTCACCT-3′) complementary to positions 887 to 871 and the oligonucleotide 5′-CCGCATCCGCGCGATAG-3′ complementary to positions 986 to 970, respectively, were used for ^{35}S labeling. For all mapping experiments, 25 μg of RNA was mixed with the labeled DNA fragments (10^7 cpm/μg of DNA).

Polymerase Chain Reaction—Polymerase chain reaction amplifications were performed in 100-μl volumes according to Sambrook *et al.* (20) in an Omnigene thermocycler (Hybaid Ltd., Teddington, U. K.) with Vent polymerase (New England Biolabs GmbH, Schwalbach, Germany). The following oligonucleotides were used as primers to amplify the coding region of *phaG* to construct plasmids pBHR-QG (derivative of pQE60 (Qiagen), insertion into *NcoI/BamHI* sites) and pBHR81 (derivative of pBBR1MCS-2 (28), insertion into *EcoRI/BamHI* sites), respectively: 5′-CATGCCATGGGAAGGCCAGAAATCGCTGTA-3′, 5′-

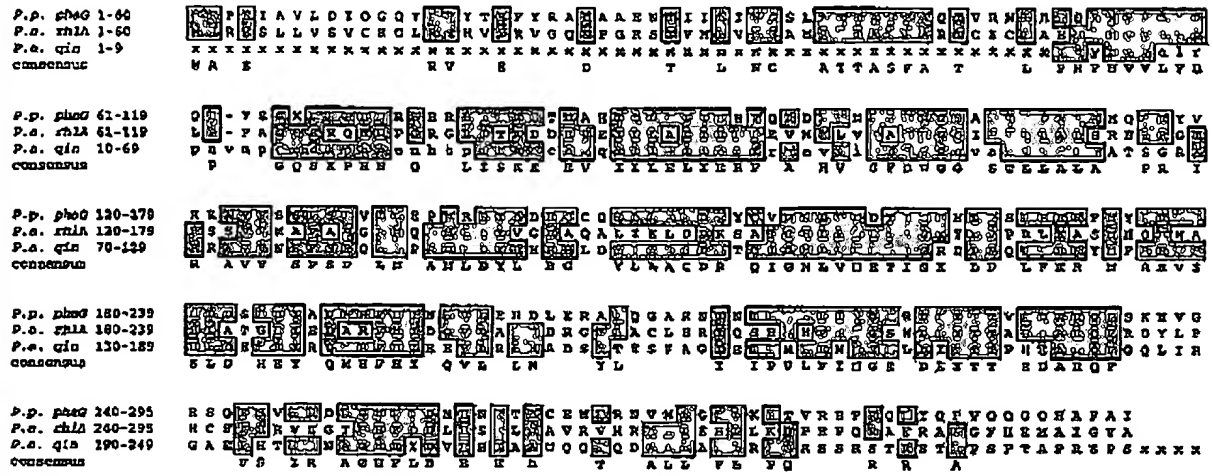

FIG. 2. **Homology of the *phaG* gene product to RhlA (40) and the putative *qin* gene product (GenEMBL data library, accession number L02105) of *P. aeruginosa*.** That part of the amino acid sequence that was deduced from the improved open reading frame analysis of the *qin* nucleotide sequence is given in *lowercase letters*. Matching amino acids are *boxed*. *Dashes* indicate gaps, which were introduced to improve the alignment. *Numbers* indicate the positions of the amino acids in the respective proteins.

CGCGGATCCGATGGCAAATGCATGCTGCCC-3' (pBHR-QG); 5'-CGCAATTCAAGGAGTCGATGACATG-3', 5'-CGCGGATCCCGGCGCCCCGTGGCC-3' (pBHR81). Both plasmids possess artificial ribosome binding sites conserved for *E. coli*, and transcription is regulated by the *lac* promoter.

Preparation of Cell Extracts and Electrophoretic Methods—Approximately 1 g (wet weight) of *E. coli* cells were suspended in 1 ml of buffer A (50 mM Tris hydrochloride, pH 7.4, 0.8% (v/v) Triton X-100, 10 mM $MgCl_2$, 10 mM EDTA, which was supplemented with 200 μg of phenylmethylsulfonyl fluoride per ml) and disrupted by sonification for 1 min at an amplitude of 14 μm in a W 250 sonifier (Branson Schallkraft GmbH, Germany). Soluble cell fractions were obtained as supernatants from 30 min of centrifugation at 50,000 × *g* and 4 °C. SDS- and mercaptoethanol-denatured proteins were separated in 11.5% (w/v) polyacrylamide gels in Tris-glycine buffer (25 mM Tris, 190 mM glycine, 0.1% (w/v) SDS (29) and stained with Coomassie Brilliant Blue (30).

Purification of Recombinant PhaG-His Tag and PhaC—Recombinant PhaG-$(His)_6$ tag (C-terminal fusion) was purified from *E. coli* JM109-harboring plasmid pBHR-QG. Crude extract was subjected to Ni^{2+}-nitrilotriacetic acid-agarose and washed twice with 20 mM imidazole, and the PhaG-$(His)_6$ tag was eluted with 250 mM imidazole. Purified PhaG-$(His)_6$ tag was used to raise anti-PhaG antibodies. Native PhaG was purified from *Pseudomonas oleovorans* ATCC 29347-harboring plasmid pBHR81 by native preparative PAGE (14% (w/v) polyacrylamide) applying the PrepCell 491 (Bio-Rad).

Analysis of (R,S)-3-Hydroxyacyl-CoA or ACP Thioester by High Performance Liquid Chromatography (HPLC)—As a reference substance, (*R,S*)-3-hydroxydecanoyl-CoA was synthesized using 10 milliunits of acyl-CoA synthetase (Sigma) in 100 μl of 50 mM Tris-HCl, pH 7.5, containing 2 mM ATP, 5 mM $MgCl_2$, 2 mM coenzyme A, and 2 mM (*R,S*)-3-hydroxydecanoate. The reaction was stopped by the addition of 6 volumes of Dole's reagent (80% (v/v), 20% (w/v) *n*-heptane, 0.02 N H_2SO_4), and remaining free fatty acid was extracted with *n*-heptane. (*R,S*)-3-Hydroxydecanoyl-ACP was synthesized as described by Rock and Cronan were used (31). HPLC analysis was conducted with a RP18 column (nucleosil C18, 7 μm, Knauer) and 25 mM potassium phosphate buffer pH 6.3 as mobile phase. Thioesters were eluted with increasing acetonitrile gradient and detected with a diode array detector (DAD 540, Kontron) at a spectral range of 200 to 500 nm with a 0.8-nm spectral resolution.

Assay of Transfer of 3-Hydroxydecanoate from CoA to ACP—The transferase assay was conducted in 100 μl of 50 mM Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$, 2 mM dithioerythrol, 500 μM acyl carrier protein (Sigma), and 2 mM (*R,S*)-3-hydroxydecanoyl-CoA with a 100-μg protein of crude extract or 50 μg of purified PhaG. After incubation for 4 h at 37 °C, the reaction was stopped by the addition of Dole's reagent, and the reaction mixture was analyzed by HPLC.

RESULTS

Complementation of Mutants Effected in the PHA Synthesis via de Novo Fatty Acid Biosynthesis—Mutants of *P. putida* KT2440, which are only deficient in the metabolic route-linking fatty acid *de novo* synthesis, were generated with nitrosoguanidine according to Miller *et al.* (16). Five mutants ($PhaG_N$) were identified, which accumulated PHA only up to 3% of the cellular dry weight (CDW) from gluconate but up to 85% PHA of CDW when cultivated on octanoate as the sole carbon source. The composition of the polymer was not affected. We constructed a library of *Eco*RI-digested *P. putida* KT2440 genomic DNA with the cosmid vector pVK100 (32) and the Gigapack II Gold Packaging Extract (Stratagene Cloning Systems, La Jolla, CA) in *E. coli* S17-1. Approximately 5,000 transductants were applied to minicomplementation experiments, with mutant $PHAG_N$-21 as recipient. One of the hybrid cosmids (pVK100::K18) harbored three *Eco*RI-fragments (3, 6, and 9 kbp) and enabled $PHAG_N$-21 to accumulate PHA from gluconate. Subcloning revealed that the 3-kbp *Eco*RI fragment (E3, pMPE3) complemented $PHAG_N$-21 and any other $PHAG_N$ mutant exhibiting this phenotype. Complementation was not achieved by the hybrid cosmid pHP1016::PP2000 comprising the entire 7.3-kbp PHA synthase locus of *P. aeruginosa* PAO1 plus approximately 13 kbp of the upstream region or by the hybrid cosmid pHP1016::PP180 comprising the *phaC2* gene of *P. aeruginosa* PAO1 plus approximately 16 kbp of the adjacent downstream region (10).

Determination of the Gene Locus and Nucleotide Sequence of phaG—Fragment E3 was cloned into pBluescript SK, and the entire nucleotide sequence was determined (Fig. 1). It comprised 3,061 nucleotides with three ORFs (Fig. 1). The only ORF that was completely localized on this fragment was ORF2 with 885 nucleotides starting at position 911 and terminating at position 1795 (Fig. 1). ORF2 will be referred to as *phaG*. A putative S/D sequence was identified eight nucleotides upstream of the start codon. About 230 bp downstream of the translational stop codon a potential factor-independent transcription terminator was located (Fig. 1). ORF1 and ORF3 are localized only incompletely on E3 with ORF1 lacking the 5'-region and with ORF3 lacking the 3'-region. The amino acid sequence deduced from ORF1 revealed significant homologies

TABLE I
Bacterial strains and plasmids

Strains and plasmids	Relevant characteristics	Source or reference
Strains		
P. putida		
KT2440	mt-2, *hsdR1* (r^-m^+), ohne TOL plasmid	(45)
$PHAG_N$-21	*P. putida* KT2440 mutants	This study
P. oleovorans	OCT plasmid	ATCC 29347
P. aeruginosa	Prototroph, Alg^-	ATCC 15692
E. coli		
S17-1	*recA*; harbors the *tra* genes of plasmid RP4 in the chromosome; *proA*, *thi-1*	(46)
JM109	*recA1 endA1 gyrA96 thi hsdR17* (rk^-,mk^+) *supE44 relA1*, λ-, *lac* [F'*proAB lacIqZ*ΔM15]	(21)
Plasmids		
pHP1016::PP180	Tc^r, Km^r, $phaC2_{Pa}$, $phaD_{Pa}$, ORF4 orientation of the Cm promoter antilinear to *phaC2*	(47)
pHP1014::PP2000	Tc^r, Km^r, $phaC1_{Pa}$, $phaZ_{Pa}$, ORF1, ORF2, $phaD_{Pa}$, ORF4'	(47)
pVK100	Tc^r, Km^r, broad host range cosmid	(32)
pVK100::K18	pVK100 harboring three genomic *Eco*RI fragments of *P. putida* KT2440 harboring *phaG*	This study
pMP92	Tc^r, broad host range plasmid	(48)
pMPE3	pMP92 containing the 3-kbp E3 fragment harboring *phaG*	This study
pUCP27	Tc^r, broad host range plasmid, *tacPOZ'*	(49)
pBHR75	pUCP27 containing the 1.3-kbp *Bam*HI-*Hin*dIII subfragment of E3 comprising *phaG* including the native promoter	This study
pMPSE22	pMP92 containing the 2.2-kbp *Sal*I-*Eco*RI subfragment of E3 harboring *phaG* without promoter	This study
pBBR1MCS-2	Km^r, broad host range, *lacPOZ'*	(28)
pBHR81	pBBR1MCS-2 containing coding region of *phaG* downstream of *lac* promotor	This study
pBluescript SK⁻	Ap^r, *lacPOZ'*, T7 and T3 promoter	Stratagene
pBluescript SK⁻BH13	pBluescript SK⁻ containing 1.3-kb *Bam*HI-*Hin*dIII subfragment of E3 comprising *phaG* including the native promoter	This study
pQE60	Ap^r, *lacP*, C terminal His tag fusion	Qiagen
pBHR-QG	pQE60 containing coding region of phaG in *Nco*I/*Bam*HI site in-frame to create His tag fusion	This study

to a hypothetical, not further characterized protein of *Hemophilus influenzae* (33). In contrast, the amino acid sequence deduced from ORF3 did not reveal any significant homology to proteins available from EMBL data base. Several other smaller ORFs were detected. However, none of them did obey the rules of Bibb *et al.* (34) for a coding region or was preceded by a reliable ribosomal binding site.

Characterization of the phaG Translational Product—The codon usages in *phaG*, ORF1 and ORF3 agreed well with typical *P. putida* codon preferences. The G + C content of 59.2 mol % for *phaG* was similar to the value of 60.7 to 62.5 mol % determined for total genomic DNA of *P. putida* (35). The *phaG* gene encodes a protein of 295 amino acids with a molecular mass of 33,876 Da. Sequence alignments of the amino acid sequence deduced from *phaG* revealed a 44% overall identity to the *rhlA* gene product of *P. aeruginosa* PG201 (Fig. 2). RhlA also consists of 295 amino acids and has a molecular mass of 32.5 kDa. This gene represents the 5'-terminal gene of a gene cluster consisting of the genes *rhlA*, *rhlB*, and *rhlR*. The first two genes encode proteins involved in rhamnolipid biosynthesis. The *rhlB* gene product exhibited rhamnosyltransferase activity, whereas the function of RhlA is not yet characterized but is necessary for effective rhamnolipid biosynthesis. RhlR represents a transcriptional activator acting upon σ^{54}-dependent promoters (36). The C-terminal regions of RhlA and PhaG revealed high homology to a gene region (*qin*) of *P. aeruginosa* encoding the so-called "quinolone-sensitivity protein" (GenEMBL data library, accession number L02105) amounting to 50.6 and 40.1% to PhaG or to RhlA, respectively, in 249 overlapping residues (Fig. 2). This region comprises 1503 nucleotides. The N terminus of the *qin* gene was not exactly determined, and the homology as depicted in the data base extents only from nucleotide 207 to 566 of this sequence (Fig. 2). However, translation of this sequence in all six reading frames and a subsequent tBLASTn search resulted in the identification of homologies also in the upstream region of the suggested *qin* translational start codons but in different reading frames with the N-terminal region of PhaG and RhlA.

Identification and Regulation of the Promoter—244 bp upstream of *phaG*, a putative σ^{70}-dependent promoter structure TTGCGCN$_{17}$TTGAAT (where N is a nucleoside) was identified. The promoter was verified by complementation studies of mutant $PHAG_N$-21 with subfragments of E3. The 2.2-kbp *Sal*I-*Eco*RI subfragment (SE 22, pMPSE22) (Fig. 1, Table I), which lacked the above-mentioned promoter sequence, did not complement this mutant, whereas the 1.3-kbp *Bam*HI-*Hin*dIII subfragment (BH13, pBHR75) (Fig. 1, Table I) of E3 conferred the ability to again synthesize PHA from simple carbon sources. In addition, the significance of this putative promoter structure was proved by S_1 nuclease protection with total RNA isolated from gluconate-grown and octanoate-grown cells of *P. putida* KT2440 harvested in the stationary growth phase. The transcriptional start site was identified 5 nucleotides downstream of the putative promoter consensus sequence at position 673 (Fig. 1, 3). For octanoate-grown cells only an extremely weak RNA signal was detected, whereas a strong signal occurred with RNA isolated from gluconate-grown cells (Fig. 3). This indicated a strong transcriptional induction of *phaG* under conditions of PHA synthesis via fatty acid *de novo* biosynthesis.

Heterologous Overexpression of phaG in E. coli—A plasmid expressing a C-terminal His(6) tag fusion protein of PhaG was constructed. The resulting plasmid pBHR-QG enabled overexpression of *phaG* under *lac* promoter control in *E. coli* JM109 (Fig. 4). The fusion protein could only be purified under denaturing conditions by immobilized metal ion affinity (Fig. 5) and was used as antigen to raise antibodies.

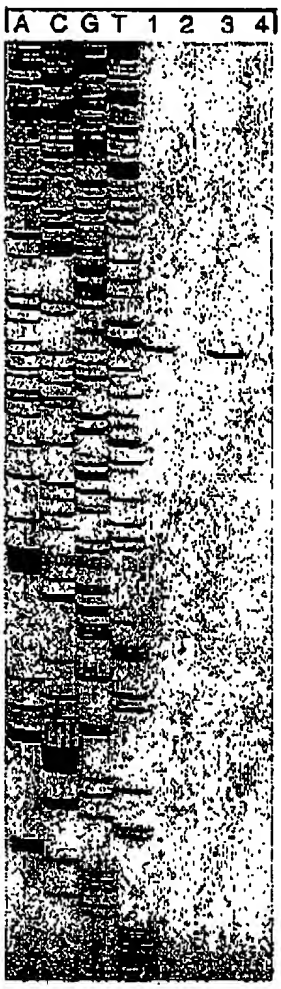

FIG. 3. S_1 nuclease protection assays of the *phaG* transcripts. *Lanes A, C, G,* and *T,* standard sequencing reactions to size the mapping signals. RNA was isolated from gluconate-grown (*lanes 1* and *3*) or octanoate-grown (*lane 4*) cells of *P. putida* KT2440 (*lanes 1* and *3*) and *A. eutrophus* H16 (*lane 2*).

Functional Homologous and Heterologous Expression of phaG—Functional expression, as revealed by complementation of mutant $PHAG_N21$, was obtained from plasmid pBHR81, a derivative of vector pBBR1MCS-2 (28) containing the coding region of *phaG* in sites *EcoRI/Bam*HI (Fig. 4, Table II). Additionally, transfer of pBHR81 into *P. oleovorans* ATCC 29347, which is not capable of PHA synthesis from simple carbon sources, resulted in PHA accumulation from gluconate contributing to about 55% of CDW (Table II). Thus only functional expression of *phaG* in *P. oleovorans* established a metabolic link between fatty acid *de novo* biosynthesis and PHA synthesis. Expression of *phaG* in *P. aeruginosa* PAO1 based on plasmid pBHR81 revealed an ~40% increase in PHA accumulation (Table II). We also investigated functional expression of *phaG* in *E. coli* JM109-harboring plasmids pBHR81 and pBHR71 allowing functional expression of PHA synthase gene *phaC1* (8), but no PHA accumulation was observed when cells were grown on glucose. Furthermore, transfer of pBHR81 into *P. aeruginosa* UO299 (*rhlA*) did not result in complemention of this mutant with respect to rhamnolipid synthesis (data not shown). Thus PhaG does not functionally replace RhlA. To evaluate whether PhaG exhibits PHA synthase activity, we cultivated the *P. putida* $PHAG_N$ mutants harboring pBHR81 under nonlimited nitrogen conditions, which resulted in decreased PHA synthase levels and decreased PHA accumulation (37). No increase in PHA accumulation was observed when cells were grown on gluconate in the presence of PhaG (data not shown).

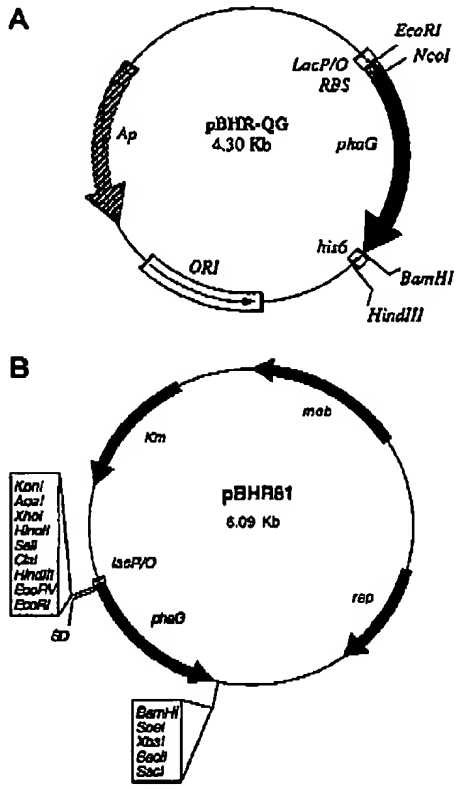

FIG. 4. **Restriction maps of plasmids pBHR-QG (*a*) and pBHR81 (*b*).** *kb,* kilobases.

Enzymatic Assay of PhaG—Native PhaG was purified from crude extracts of *P. oleovorans* (pBHR81) by native PAGE as described under "Experimental Procedures." Recombinant PhaG showed high mobility in native PAGE, which could be utilized for one-step purification (Fig. 5). PhaG was also identified by N-terminal amino acid sequencing.

Purified PhaG and Crude Extracts from *P. oleovorans* (pBHR81) were employed to demonstrate enzymatic activity of PhaG. As substrate we provided *in vitro* synthesized (*R,S*)-3-hydroxydecanoyl-CoA and analyzed the reaction products by HPLC (Fig. 6). *P. oleovorans* harboring only vector pBBR1MCS-2 and heat-inactivated purified PhaG served as negative control. The HPLC data clearly demonstrate that, applying either crude extract or purified PhaG, a transfer of the 3-hydroxydecanoyl moeity from CoA to ACP occurs (Fig. 6). The omission of $MgCl_2$ resulted in a loss of enzymatic activity, indicating that $MgCl_2$ is an important cofactor. Furthermore, we applied the straight chain octanoyl-CoA and decanoyl-CoA thioesters as substrate. None of these CoA thioesters yielded the corresponding ACP thioester, and they were therefore not accepted as substrate by PhaG.

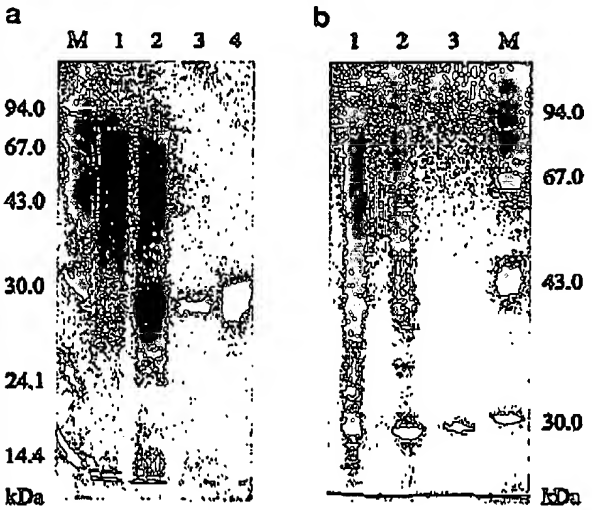

FIG. 5. *a*, heterologous expression of *phaG-His tag* in *E. coli* and purification. Cytoplasmic fractions obtained from cells of recombinant strains of *E. coli* grown in LB medium and fractions from batch purification with Ni^{2+}-nitrilotriacetic acid-agarose were separated in 11.5% (w/v) polyacrylamide gels and stained to visualize protein with Serva blue R. *M*, molecular weight standards. *Lane 1*, crude extract of *E. coli* JM109 (pQE60); *lane 2*, crude extract of *E. coli* JM109 (pBHR-QG); *lane 3*, eluate after washing with 20 mM imidazole; *lane 4*, purified PhaG-His tag after elution with 250 mM imidazole. *b*, heterologous expression of *phaG* in *P. oleovorans* and purification of native PhaG. *P. oleovorans* harboring pBHR81 was cultivated 16 h at 30 °C on mineral salts medium containing 1% (w/v) gluconate. Crude extracts were applied to native PAGE (PrepCell 491, Bio-Rad), and the first fraction with high absorption at 280 nm yielding purified PhaG was analyzed. *M*, molecular weight standards. *Lane 1*, crude extract of *P. oleovorans* (pBBR1MCS-2); *lane 2*, crude extract of *P. oleovorans* (pBHR81); *lane 3*, first protein eluate from native PAGE containing pure PhaG.

DISCUSSION

Phenotypical complementation of *P. putida* KT2440 $PHAG_N$ mutants, which are affected in PHA biosynthesis based on fatty acid *de novo* biosynthesis, led to the identification and characterization of *phaG* as a new gene locus relevant for PHA biosynthesis in *P. putida*. The PHA synthesis pathway via β-oxidation was not impaired in the $PHAG_N$ mutants. $PHAG_N$ mutants were not complemented with the PHA synthase locus of *P. aeruginosa* PAO1 and adjacent genomic region. Therefore, $PHAG_N$ mutants are not defective in the PHA synthase locus, and most probably *phaG* is not closely linked to the PHA synthase locus. Furthermore, *phaG* is not in general essential for the synthesis of PHA in *P. putida* KT2440 but is only required for PHA synthesis and accumulation from gluconate or other simple carbon sources, which are catabolized to acetyl-CoA in this organism before PHA synthesis starts.

From results of labeling studies, nuclear magnetic resonance spectroscopy and gas chromatography-mass spectroscopy Eggink *et al.* (4) and Huijberts *et al.* (7, 38) concluded that the precursors of PHA_{MCL} biosynthesis from simple carbon sources are predominantly derived from (*R*)-3-hydroxyacyl-ACP intermediates occurring during the fatty acid *de novo* biosynthetic route. Since the constituents of PHB and PHA represent the *R* configuration, and since PHA_{SCL} and PHA_{MCL} synthases are highly homologous, the intermediates in fatty acid metabolism are presumably converted to (*R*)-3-hydroxyacyl-CoA before polymerization. Nevertheless, some other routes of PHA synthesis are also possible. Other conceivable alternatives are the release of free fatty acids by the activity of a thioesterase with a thiokinase, subsequently activating these fatty acids to the corresponding hydroxyacyl-CoA thioesters or chain elongation with β-ketothiolase, or β-oxidation of synthesized fatty acids. Evidence for the latter pathways in *P. putida* (7) was obtained and explains why *phaG* mutants are not completely defective in PHA_{MCL} biosynthesis from gluconate. Functional expression of either PHA synthase and accumulation of PHA_{MCL} from fatty acids indicate that PHA synthases are not utilizing (*R*)-3-hydroxyacyl-ACP derivatives as substrate (8, 9).

All mutants analyzed and complemented by *phaG* synthesized PHA to some extent (0.5–3% CDW) with a typical monomer composition of polyester derived from simple carbon sources, as far as detectable. However, analysis of mutant complementation studies and the genomic organization of *phaG* revealed no indication for the existence of another protein essential for the PHA synthesis from simple carbon sources in *P. putida* KT 2440. Therefore, most probably only one additional specific enzymatic step is required for PHA synthesis from gluconate that is not required for PHA synthesis from octanoate. This hypothesis was supported by the observation that only PhaG conferred the ability to synthesize PHA from gluconate to *P. oleovorans*, which lacks this capability (Table II). Furthermore, the analysis of enzymatic activity of PhaG strongly suggests that one enzyme is sufficient to link fatty acid *de novo* synthesis with PHA synthesis (Fig. 6). Evidence that PhaG is not directly involved in synthesis of PHA_{MCL} was provided by cultivations of the *P. putida* $PHAG_N$ mutants (pBHR81) under nitrogen limited and nonlimited conditions. Under nonlimited conditions the level of PHA synthases and PHA_{MCL} accumulation is significantly decreased (37), and even in the presence of PhaG, no increase in PHA_{MCL} synthesis was observed.

Although no complementation of rhamnolipid synthesis in *P. aeruginosa rhlA* mutant UO299 was obtained with *phaG* expressed from plasmid pBHR81, the high degree of homology of *phaG* to *rhlA* and the *qin* region of *P. aeruginosa*, respectively, indicates a related function of these proteins. The exact function of the "quinolone sensitivity protein" has not yet been described. Quinolones such as nalidixic acid are synthetic antibiotics exhibiting strong antimicrobial effects on Gram-negative bacteria including *P. aeruginosa*. The *rhlA* gene product is involved in the rhamnolipid biosynthesis of *P. aeruginosa* PG201, which are synthesized as biosurfactants during the late exponential and stationary growth phases. Rhamnolipid biosynthesis proceeds by sequential glycosyl transfer reactions, each catalyzed by specific rhamnosyltransferases with TDP-rhamnose acting as a rhamnosyl donor, and 3-hydroxydecanoyl-3-hydroxydecanoate or L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate acting as acceptors as proposed by Burger *et al.* (39, 40). 3-Hydroxydecanoate can be formed via β-oxidation or via fatty acid *de novo* biosynthesis (41). A dimer consisting of two 3-hydroxydecanoic acid molecules is formed by a hitherto unknown mechanism. RhlA significantly enhanced the level of rhamnolipids in rhamnolipid-negative mutants of *P. aeruginosa* PG201 when it was coexpressed with the rhamnosyltransferase (RhlB) as compared with the expression of the isolated *rhlB* gene.

3-Hydroxyacyl-ACP intermediates provided by fatty acid biosynthesis are presumably the common intermediates of PHA and rhamnolipid biosynthesis from gluconate. If the ACP derivatives themselves do not serve as substrates for PHA synthases or enzymes involved in rhamnolipid synthesis for the condensation of two 3-hydroxydecanoyl moieties, they must be either directly transesterified to the corresponding CoA derivatives or transferred to CoA thioesters by the combined action of a thioesterase and a thiokinase. Various transacylases and acyltransferases have been described and well characterized

TABLE II

Complementation of P. putida mutant PHAG$_{Pp}$21 and functional heterologous expression of phaG in various pseudomonads

PHA content and comonomer composition of various pseudomonads harboring either vector pBBR1MCS-2 or pBHR81. Cells were grown for 48 h at 37 °C (*P. aeruginosa*) or at 30 °C (all others). Cultivations were performed in a mineral salts medium containing 1% (w/v) gluconate. PHA content and comonomer composition were analyzed. 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate.

Strain	Plasmid	PHA content	Composition of PHA			
			3HHx	3HO	3HD	3HDD
		% (w/w) CDW	mol %			
P. putida KT2440	pBBR1MCS-2	54	3.1	24.2	66.4	6.3
	pBHR81	60	3.2	14.2	75.1	7.5
P. putida PHAG$_{Pp}$21	pBBR1MCS-2	3	"ND"[a]	25.3	65	10
	pBHR81	50	3.1	14.2	76.6	6.1
P. aeruginosa PAO1	pBBR1MCS-2	37	2.5	20.5	68	9
	pBHR81	51	2.6	25	60	12.4
P. oleovorans[b]	pBBR1MCS-2	3	ND	ND	75	25
	pBHR81	46	1	7.5	78	13.5

[a] ND, not detectable.
[b] Strain ATCC29347.

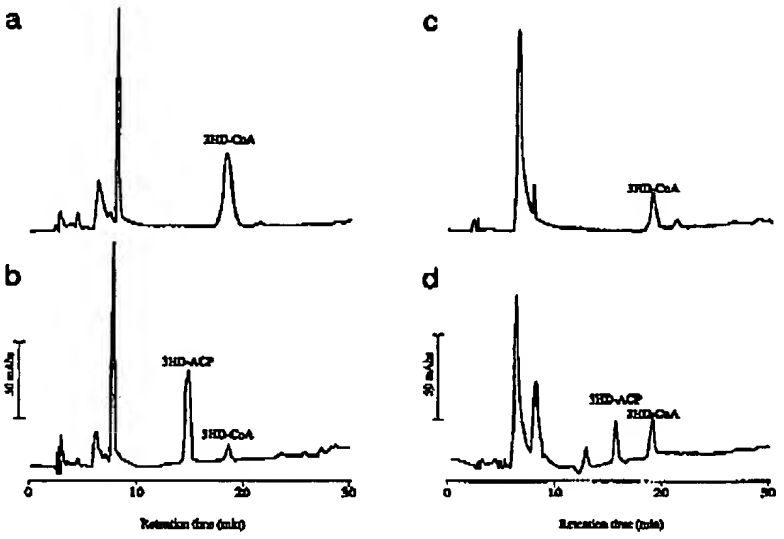

FIG. 6. **HPLC analysis of reaction products from enzymatic assay with PhaG.** *a*, crude extracts from various bacteria harboring either (*a*) vector pBBR1MCS-2 (negative control) or (*b*) plasmid pBHR81 were employed for the enzymatic PhaG assay. *d*, purified PhaG was directly used for the assay (*c*) with heat-inactivated PhaG as negative control. 3-Hydroxydecanoyl-CoA (*3HD-CoA*) was provided as substrate, and the transfer of the acyl moeity to ACP was demonstrated (3-hydroxydecanoyl-ACP (*3HD-ACP*)). Peaks were identified based on their R_f values, by co-chromatography, and by their spectra. The identity of relevant peaks was indicated.

catalyzing the direct transfer of an acyl moiety, e.g. (i) the malonyl-CoA-ACP transferase, which catalyzes the transfer of the malonyl moeity from CoA to ACP (12) and (ii) the hydroxydecanoyl-ACP-dependent UDP-GlcNAc acyltransferase, which catalyzes the transfer of hydroxydecanoyl moeity from ACP to UDP-GlcNAc (13, 14). The bacterial acyltransferase LpxA is one representative of a large family that possesses conserved repeating hexapeptides (42). Sequence analysis of membrane-bound glycerolipid acyltransferases revealed that these proteins share a highly conserved domain containing invariant histidine and aspartic acid residues separated by four less conserved residues in an HX_4D configuration (43). Site-directed mutagenesis of the invariant histidine resulted in lack of activity, indicating an essential role of this residue (43). Although no significant homology of PhaG to transacylases and acyltransferases was found, this highly conserved HX_4D mini-motif is also present in PhaG at positions 176–181 of the amino acid sequence (Fig. 1), suggesting a similar function of PhaG. The studies on heterologous expression of *phaG* and the enzymatic characterization of PhaG strongly suggests that PhaG catalyzes the conversion of (*R*)-3-hydroxyacyl-ACP to (*R*)-3-hydroxyacyl-CoA derivatives (Table II, Fig. 6), which serve as ultimate precursors for the PHA polymerization from unrelated substrates in pseudomonads proposed recently (4, 44).

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